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Generation, characterization and crystallization of a highly active and stable cytochrome bc_1 complex mutant from *Rhodobacter sphaeroides*

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Abstract

The availability of the three dimensional structure of mitochondrial enzyme, obtained by X-ray crystallography, allowed a significant progress in the understanding of the structure–function relation of the cytochrome bc_1 complex. Most of the structural information obtained has been confirmed by molecular genetic studies of the bacterial complex. Despite its small size and simple subunit composition, high quality crystals of the bacterial complex have been difficult to obtain and so far, only low resolution structural data has been reported. The low quality crystal observed is likely associated in part with the low activity and stability of the purified complex. To mitigate this problem, we recently engineered a mutant [S287R(cyt b)/V135S(ISP)] from *Rhodobacter sphaeroides* to produce a highly active and more stable cytochrome bc_1 complex. The purified mutant complex shows a 40% increase in electron transfer activity as compared to that of the wild type enzyme. Differential scanning calorimetric study shows that the mutant is more stable than the wild type complex as indicated by a 4.3 °C increase in the thermo-denaturation temperature. Crystals formed from this mutant complex, in the presence of stigmatellin, diffract X-rays up to 2.9 Å resolution.

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1. Introduction

The cytochrome bc_1 complex, also known as ubiquinol cytochrome c reductase, is a multi-subunit integral membrane protein that constitutes an essential component of the cellular respiratory chain. It catalyzes electron transfer from ubiquinol to cytochrome c coupled with proton translocation across the membrane [1]. The electrochemical gradient generated is used as an energy source to drive ATP synthesis by ATP synthase. The bc_1 complex is com-

posed of varying number of subunits ranging from three subunits, as in *Paracoccus denitrificans* and *Rhodobacter capsulatus* [2,3], to as many as eleven subunits, as in bovine mitochondria [4]. The three main redox subunits, cyt b housing hemes b_L and b_H , cyt c_1 housing heme c and Iron sulfur protein (ISP) housing the iron sulfur cluster [2Fe–2S], are found in all species and are directly involved in the electron transfer mechanism from ubiquinol to cyt c as defined by the Q cycle theory [5,6]. All additional subunits, referred to as supernumerary subunits, are believed to be involved in the increased stability of the protein [7,8]. Despite its size (247 kDa) and complexity (11 subunits), the crystal structure of bovine mitochondrial cyt bc_1 complex [9] was reported in 1997, and several crystallographic studies were done afterwards to understand the structural aspects of the binding environment of the quinone reduction (Qi) and quinol oxidation (Qo) sites using substrate ubiquinone and several Qo and Qi binding inhibitors [10,11]. The cyt bc_1 complex isolated from *Rb. sphaeroides* contains the three redox-active subunits and only one supernumerary subunit — Subunit IV [8]. The simpler subunit composition of the bacterial complex and the ability to express and purify lethal protein from *Rb. sphaeroides* make it an excellent

Abbreviations: bc_1 complex, ubiquinol-cytochrome c oxidoreductase; b_H , higher potential cytochrome b heme; b_L , lower potential cytochrome b heme; Cyt, cytochrome; DSC, differential scanning calorimetry; EDTA, Ethylenediaminetetraacetic acid; *E. coli*, *Escherichia coli*; ISP, Rieske iron–sulfur protein; LB, Luria Broth; LM, N-Dodecyl- β -D-maltoside; β -ME, β -mercaptoethanol; Ni-NTA, Nickel-nitrilotriacetic acid; OG, n-octyl- β -D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; Q, ubiquinone; Q₀C₁₀Br, 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol; *Rb*, *Rhodobacter*; SDS, sodium dodecyl sulfate; Tm, melting temperature; [2Fe–2S], Rieske iron–sulfur center

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model of the mitochondrial complex and an ideal system to understand the mechanism of electron transfer. Most of the structural information obtained from the mitochondrial enzyme has been validated via molecular genetic manipulation of the bacterial enzyme. Although the structure of the bacterial complex is expected to be very similar to that of mitochondrial complex, a real structure of the bacterial enzyme is needed if more detailed structure and a mechanism of catalytic function of the cyt *bc*₁ complex is to be explored. Despite of the simplicity of the *Rb. sphaeroides* cyt *bc*₁ complex, no high quality crystals have been obtained yet. *Rb. sphaeroides* cyt *bc*₁ complex was crystallized in our laboratory over 3 years ago but the crystals could only diffract anisotropically to 3.8 Å in the best direction, to the same resolution *Rb. capsulatus* cyt *bc*₁ complex structure was reported [12]. We believe that at this resolution, the differences between the mitochondrial and the bacterial systems cannot be clear enough to make definite conclusions. The low quality crystal observed is most likely associated with the low activity and stability of the purified complex. Herein, we report an engineered mutant of *Rb. sphaeroides* cyt *bc*₁ complex, the SR mutant – SuperReductase – that grows photosynthetically at a similar rate to that of wild type but conspicuously shows higher enzymatic activity in chromatophore membranes and in purified proteins. It also shows a significant increase in the purified protein stability as indicated by the increased thermo-denaturation temperature determined by differential scanning calorimetry. The SR mutant crystallization yielded small red translucent crystals that diffract to 2.9 Å in the presence of stigmatellin. The reported mutant is believed to be an excellent system to improve and accelerate crystallographic studies to elucidate all structural information needed of the cyt *bc*₁ complex.

2. Materials and methods

Cytochrome *c* (from horse heart, type III) was purchased from Sigma. N-Dodecyl-β-D-maltoside (LM) and N-octyl-β-D-glucoside (OG) were from Anatrace. Stigmatellin was purchased from Fluka. Ni-NTA gel and Qiaprep Spin Miniprep Kit were from Qiagen. 2, 3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1, 4-benzoquinol (Q₀C₁₀BrH₂) was prepared in our laboratory as previously reported [13].

2.1. Growth of bacteria

E. coli cells were grown at 37 °C in LB medium. *Rb. sphaeroides* BC17 cells bearing pRKD418-*fb*C_{6H}Q [14] plasmid were grown photosynthetically at 30 °C in an enriched sistrom medium containing 5 mM glutamate and 0.2% casamino acids. Photosynthetic growth conditions for *Rb. sphaeroides* were essentially as described previously [15]. The growth of *Rb. sphaeroides* strains was monitored by measuring O.D₆₀₀ every 3–5 h. Cells were transferred to a larger batch or harvested when O.D₆₀₀ reached 1.8–2.0.

2.2. Antibiotics were added to the following concentrations

125 µg/mL ampicillin, 20 µg/mL Kanamycin sulfate, 25 µg/mL Trimethoprim, 10 µg/mL tetracycline for *E. coli* and 1 µg/mL for *Rb. sphaeroides*.

2.3. Generation of *Rb. Sphaeroides* expressing mutants of *bc*₁ complex

Mutations were constructed by site-directed mutagenesis using the Quick-Change system from Stratagene. The double stranded pGEM7Zf(+)-*fb*C_{6H}Q was used as a template to generate the mutant S287R(b)/V135S(ISP), referred to as the SR mutant. pGEM7Zf(+)-*fb*C_{6H}Q was constructed by ligating the *Xba*I–*Eco*RI fragment of pRKD418-*fb*C_{6H}Q [14] into the *Xba*I–*Eco*RI digested pGEM7Zf(+). The following primers were used to introduce the single mutation V135S in ISP: V135S-F 5'-CCCACCTCGGCTGCTCGCCGATCGGCGGCGTGTGTC-3' and V135S-R 5'-GACACGCCGCCGATCGGCGAGCAGCCGAGGTGGG-3'.

The double mutant S287R(b)/V135S(ISP) was constructed using pGEM7Zf(+)-*fb*C_{6H}Q carrying the single mutant V135S(ISP) as template and S287R(b) primers. S287R(b)-F 5'-GCGAACCCGCTCCGGACGCCCGCGCACATCG 3' and S287R(b)-R 5'-CGATGTGCGCGGCGTCCGGAGCGGGTTCGC-3'.

The presence of mutations in ISP and cyt *b* was confirmed by DNA sequencing which was carried out at the Recombinant DNA/Protein Core Facility at Oklahoma State University. Plasmid bearing successful mutations – S287R(b)/V135S(ISP) – was digested with *Xba*I and *Eco*RI and the generated *fb*C_{6H}Q fragment was purified and ligated to the pRKD418-C_{6H}Q vector, generated from the digestion of pRKD418-*fb*C_{6H}Q plasmid with *Xba*I and *Eco*RI. The generated pRKD418-*fb*C_{6H}Q plasmid bearing the double mutation was chemically transformed to *E. coli* S17 cells. A plate mating procedure [14] was then used to mobilize the plasmid from *E. coli* S17 into *Rb. sphaeroides* BC17.

2.4. Purification of cyt *bc*₁ complex from *Rb. sphaeroides*

Chromatophore membranes were prepared as described previously [15] and stored at –80 °C in the presence of 20% glycerol until use. Frozen chromatophores

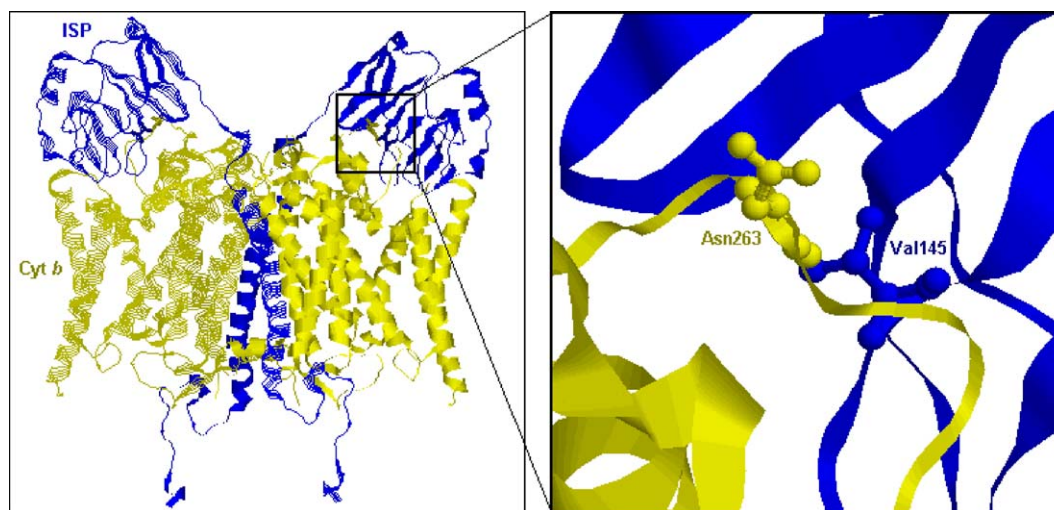


Fig. 1. Partial structure of bovine mitochondrial cytochrome *bc*₁ complex. For clarification purposes, only subunits cytochrome *b* (yellow) and ISP (blue) of each monomer of cytochrome *bc*₁ complex are shown. The close up shows the close proximity between the amino acids Asn263 (yellow) of cyt *b* and Val145 (blue) of ISP.

Table 1
Summary of the wild type and the SR mutant characteristics

Strain	Mutation	Corresponding residue in bovine	Ps growth	Enzymatic activity		Protein solet/UV ratio
				Chromatophore	Purified protein	
WT	–	–	+++	2.0	2.5	1.2
SR	S287R	N263	+++	2.8	3.5	1.3
	(cyt <i>b</i>)	(cyt <i>b</i>)				
	V135	V145				
	(ISP)	(ISP)				

Ps Growth (+++) refers to the photosynthetic growth rate of wild type cells. The enzymatic activity is expressed as $\mu\text{mol cyt } c \text{ reduced/min/nmoles cyt } b$.

were used to prepare cyt *bc*₁ complex as described previously [16]. Purified proteins were stored at -80°C in the presence of 10% glycerol.

Cyt *b* [17] and cyt *c*₁ [18] concentrations were determined spectrophotometrically as published previously. Protein concentration was determined based on absorbance at 280 nm using a converting factor of $1 \text{ O.D}_{280} = 0.56 \text{ mg/mL cm}$.

2.5. Activity assay of purified *bc*₁ complex

To assay the activity of cyt *bc*₁ complex in chromatophore membrane or intracytoplasmic membrane or in purified protein, the preparations were diluted to a final cyt *b* concentration of 3 μM with 50 mM Tris–HCl, pH 8.0, containing 200 mM NaCl and 0.01% LM. Aliquots of 2, 4 or 6 μL of the diluted sample was added to 1 mL of assay mixture containing 0.3 mM EDTA, and 100 μM cyt *c* in 100 mM Na^+/K^+ phosphate buffer, pH 7.4. The assays were started by addition of 25 μM $\text{Q}_0\text{C}_{10}\text{BrH}_2$. Activity was determined by measuring the reduction of cyt *c*, which is monitored by the increase in absorbance at 550 nm in a Shimadzu UV2102 PC spectrophotometer at 23°C , using a millimolar extinction coefficient of 18.5. For calculation purposes, the non-enzymatic oxidation of $\text{Q}_0\text{C}_{10}\text{BrH}_2$ in the absence of enzyme under these conditions was subtracted.

2.6. Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmle [19] using Bio-Rad Mini Protean dual slab vertical cell. Samples were digested with 10 mM Tris–HCl buffer, pH 6.8 containing 1% SDS and 3% glycerol in the presence or absence of 0.4% β -mercaptoethanol for 15 min at 23°C before being subjected to electrophoresis.

Differential scanning calorimetry was performed using N-DSCII. Purified protein (0.55 mL of 15 μM cyt *bc*₁ complex) was first degassed at room temperature for 10 min. Thermoscans from 10 to 90°C at a rate of 1°C/min and 2°C/min were performed during the heating and cooling scans, respectively. Three scans were recorded, heating–cooling–heating, using the third scan as a baseline for the first scan. CpCalc program was used to calculate the melting temperature (T_m) values of purified proteins.

2.7. Protein crystallization

The purified protein concentration was adjusted to 15 mg/mL with 50 mM Tris–HCl (pH 7.5) buffer containing 0.5% OG, 5 mM $\text{Na}_2\text{S}_2\text{O}_3$, 200 mM NaCl, 2 mM diheptyl phosphatidylcholine, 10% glycerol and 200 mM histidine. Stigmatellin was added to a five fold molar excess of cytochrome *bc*₁ complex and allowed to incubate on ice for 12 h followed by addition of 0.12% SMC and 10% polyethylene glycol (PEG 400). The mixture was allowed to stand overnight at 4°C . Afterwards, a small amount of precipitate was removed by centrifugation and the clear supernatant solution was used for the crystallization experiment using sitting drops method at 15°C . Small red translucent crystals appeared within 4–6 weeks of incubation. The reservoir solution contained 100 mM Tris–HCl (pH 8.0), 600 mM NaCl, 20% glycerol, 5 mM $\text{Na}_2\text{S}_2\text{O}_3$ and 26% PEG 400.

2.8. X-ray diffraction experiment and data processing

The *Rb. sphaeroides bc*₁ crystals were frozen in liquid propane and diffraction experiments were performed using synchrotron radiation source at the SER-CAT ID beamline of Advanced Photon Source at Argonne National Laboratory. The X-ray was tuned to a wavelength of 1 Å and crystals were normally exposed for about 2–5 s with an oscillation angle of 0.5° . Diffraction images were collected on a MarCCD-300 detector and were processed with HKL2000 [20]. Processing of X-ray Diffraction data were collected in Oscillation Mode. The quality of diffraction of *Rb. sphaeroides bc*₁ crystals were evaluated with the same program.

3. Results and discussion

3.1. Engineering a highly active and stable *Rb. sphaeroides* cyt *bc*₁ complex

To determine the importance of ISP head domain movement during cyt *bc*₁ catalysis, several mutants were engineered to increase the interaction between cyt *b* and ISP to immobilize the ISP head domain. Such mutants include introducing one or more disulfide bond(s) to cross-link the two subunits in the membrane domain and to fix ISP head domain in the *b*-position [21]. Upon introducing a disulfide bond between ISP head domain and cyt *b*, fixing ISP head in the *b*-position, the cyt *bc*₁ complex becomes inactive and activity can only be restored upon disruption of the disulfide bond by β -mercaptoethanol. Different sets of mutants are being designed to alter the interaction between cyt *b* and ISP to various degrees in order to study the kinetics of electron transfer between *c*₁ and ISP and to aid with ongoing structure determination of *Rb. sphaeroides* cyt *bc*₁ complex. The degree of interaction is manipulated by introducing additional hydrogen bonds between residues of the two subunits that are located in close proximity.

Based on the Bovine mitochondrial structure [9], the distance between Asn263 of cyt *b* and Val145 of ISP is close enough to allow hydrogen bonding (Fig. 1). Therefore, the corresponding residues in *Rb. sphaeroides* based on sequence alignment, Ser287 of cyt *b* and Val135 of ISP were mutated to Arg and Ser, respectively. The hydroxyl group of Serine and guanidino group of Arginine are expected to form a hydrogen bond. The cyt *bc*₁

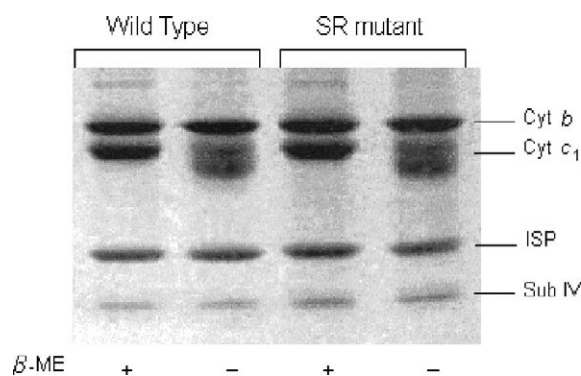


Fig. 2. SDS-PAGE analysis of the cytochrome *bc*₁ complexes isolated from wild type and SR mutant. Samples of proteins were incubated at room temperature under reducing (+ β -ME) or non-reducing (– β -ME) reducing conditions for 15 min before being subject to electrophoresis.

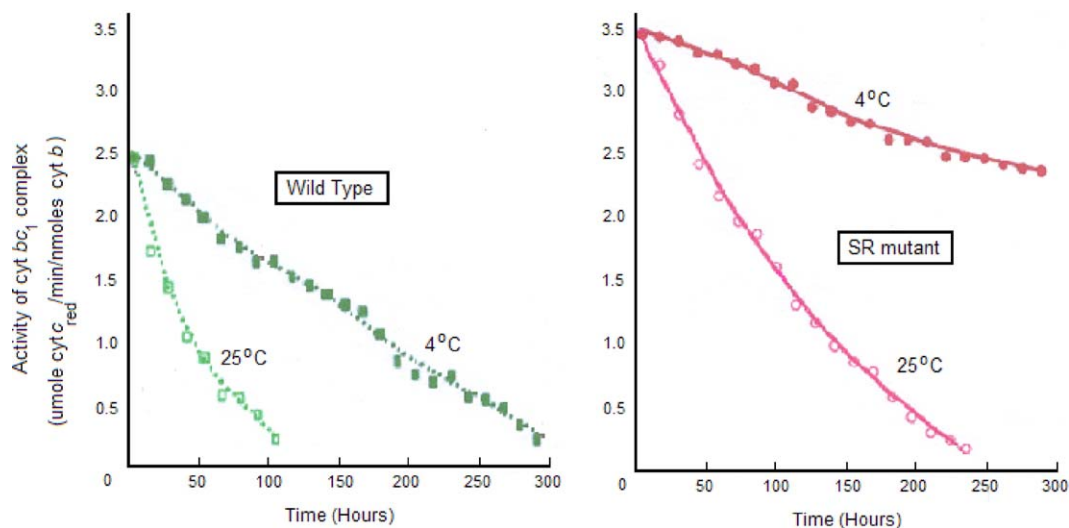


Fig. 3. Stability of purified cytochrome bc_1 complex from wild type and SR mutant at 4 °C and 25 °C.

complex double mutant S287R(b)/V135S(ISP) generated was purified and characterized to reveal the effect of increased interaction between cyt b and ISP subunits through potential hydrogen bonding.

3.2. Characteristics of the SR mutant of *Rb. sphaeroides* cyt bc_1 complex

The purified protein from the double mutant of *Rb. sphaeroides* was subject to detailed studies and compared to the wild type protein. The SR mutant grows photosynthetically at a similar rate to that of wild type. Interestingly, purified chromatophore

membranes show a 40% increase in activity as compared to the wild type protein (Table 1). The mutant protein was highly purified with a Soret to UV ratio (A_{418}/A_{280}) of 1.3 compared to a maximum value of 1.2 obtained with purified wild type protein. The mutant protein shows a 40% increase in enzymatic activity as well, compared to the wild type protein. SDS-PAGE analysis (Fig. 2) reveals no difference in subunit composition and concentration between the wild type and the mutant protein. The additional fragment observed in the absence of β -mercaptoethanol is the result of the sensitivity of cytochrome c_1 to the absence of reducing agent, which is associated with the presence of the linked c-type heme [22]. The observation of such a highly active cyt bc_1 complex obviously cannot be attributed to the better protein preparation but rather to either the genuine property of the complex or to its higher stability. A highly stable protein could be useful to improve the crystallization behavior of *Rb. sphaeroides* cyt bc_1 complex. To study the stability of the protein, the activity of the purified mutant protein was monitored over time, at 25 °C and 4 °C, and compared to the wild type activity. As shown in Fig. 3, the activity of the SR mutant decreases at a slower rate than that of the wild type protein. Within 4 days (100 h) of incubation at 25 °C, the wild type proteins become inactive whereas the SR mutant proteins still conserve about 50% of activity. Within 12 days

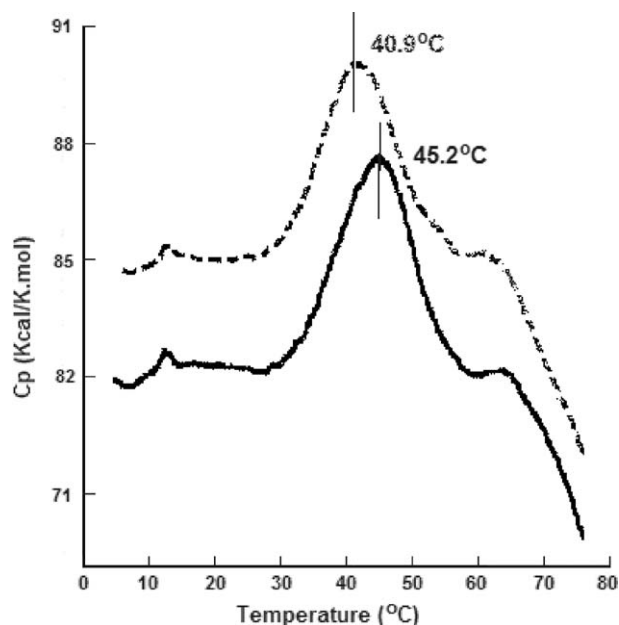


Fig. 4. Differential scanning calorimetric study of purified cytochrome bc_1 complex. The thermo-denaturation temperature of the wild type (dashed line) and SR mutant (solid line) cyt bc_1 complex is 45.2 °C and 40.9 °C, respectively. Purified protein (0.55 mL of 15 μ M cyt bc_1 complex from wild type or SR mutant) was subjected to differential scanning calorimetry as described in Materials and methods.

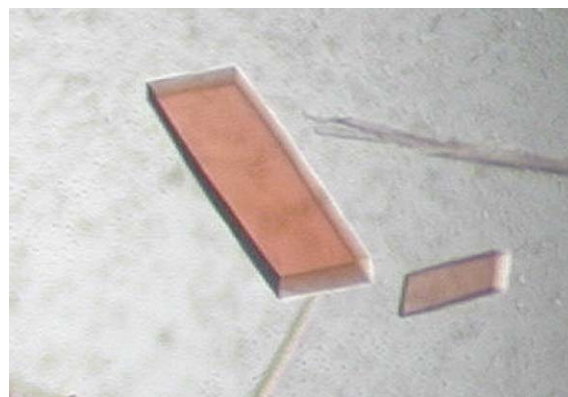


Fig. 5. Crystal of *Rb. Sphaeroides* SR mutant cytochrome bc_1 complex.

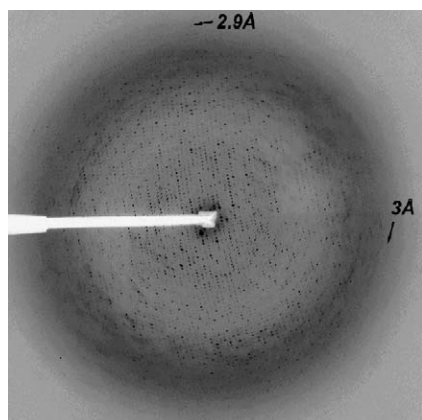


Fig. 6. Diffraction pattern of a *Rb. Sphaeroides* SR mutant cytochrome bc_1 complex crystal. The highest resolution spot by visual inspection is indicated by an arrow.

(300 h) of incubation at 4 °C, the SR mutant proteins conserve 70% of activity as compared to nearly complete inactivation of the wild type protein. To confirm this conclusion, the purified mutant protein was subjected to a differential scanning calorimetry experiment to analyze its thermo-denaturation temperature. The purified SR protein showed a 4.3 °C increase in its melting temperature as compared to the wild type protein (Fig. 4). These results suggest that the SR mutant protein is more stable than wild type protein and therefore might be better behaved in crystallization experiments.

3.3. Crystallization and preliminary X-ray diffraction analysis

A lot of effort has been devoted through the years to the crystallization of the wild type $cyt\ bc_1$ complex from *Rb. sphaeroides*. Despite of the relative small size and simpler subunit composition as compared to the mitochondrial complex, the extensive crystallization effort of the bacterial bc_1 complex could only lead to low quality crystals that diffracted X-rays anisotropically and to low resolution. As prevalent as it is in the field of membrane protein structural biology, the difficulties in crystallizing membrane protein are rooted deeply to the fact that large number of parameters affects the protein behavior during crystallization. One of these parameters and with no doubt an important one is the stability of the protein. Since the crystallization process often involves a prolonged incubation period, the observed enhancement in stability of the mutant protein is thought to offer an appealing system to screen for better conditions for *Rb. sphaeroides* $cyt\ bc_1$ crystals. Indeed, the SR mutant was crystallized in the presence of stigmatellin, which further stabilizes the protein during incubation, at 15 °C in several different crystal forms. Small, red translucent crystals were observed within 4–6 weeks (Fig. 5). More importantly, the mutant *Rb. sphaeroides* $cyt\ bc_1$ preparation allowed us to identify conditions that produced crystals diffracting X-rays uniformly to 2.9 Å resolution (Fig. 6). Analysis of crystalline complex by SDS-PAGE revealed only the three core subunits. The supernumerary subunit (Sub IV) was recovered in the mother liquid solution. Apparently, subunit IV is released from the complex upon incubation under the specified crystallization conditions. A detailed description of the structure

determination and analysis of *Rb. Sphaeroides* $cyt\ bc_1$ complex will be published elsewhere (Esser et al., in preparation).

The main challenge in membrane protein crystallization is the protein chemistry rather than crystallography itself. The lack of sufficient protein stability presents a serious barrier for crystallographers to determine a structure. Protein enhancement through mutant screening for greater stability and higher activity can lead to a major progress in structure determination. Such approach can be particularly effective when low resolution crystals are available to guide in mutant engineering.

Acknowledgment

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